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THE EFFECTS OF INHIBITORS AFFECTING PROTEIN SYNTHESIS AND MEMBRANE ACTIVITY ON THE *CHLAMYDOMONAS REINHARDII* PHOTOTACTIC RHYTHM

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Abstract

Cycloheximide and puromycin slowed the biological timing of the phototactic rhythm of *Chlamydomonas reinhardii*. However, actinomycin D, rifampicin, chloramphenicol, streptomycin, chloral hydrate, valinomycin, LiCl, ethanol, methanol, isopropanol, procaine, tetracaine, dibucaine, and sodium lauryl sulfate did not alter the period length.

A cycloheximide resistant mutant (*cyr*-1) was isolated. The mutation is recessive since diploids produced from *cyr*-1 and wild type are sensitive to cycloheximide. The phototactic rhythm of *cyr*-1, unlike that of the wild type, is not influenced by cycloheximide. This supports the view that the action of this drug in slowing the rhythm in the wild type is mediated by the inhibition of protein synthesis.

Introduction

The cellular and behavioral activities of many eucaryotic organisms are regulated by a biological clock that is separate from the activities it governs. This biological timing mechanism is adaptive because it allows organisms' activities to occur at the most propitious time, either of the day or relative to another rhythmic activity. Although many of the properties of this clock have been identified, an understanding of the molecular mechanism of the circadian clock still eludes us.

One method of attempting to decipher the mechanism of the clock has been to subject an organism to chemicals with known effects, hoping that they will alter the clockworks in a manner that will reveal some aspect of its machinery. If a drug reaches the clock and interferes with a process important to timekeeping, its sustained administration would be expected to result in a change in the period length of the observed rhythm. Because many substances inhibit cellular processes and are fatal when administered continuously, and because it is of interest to identify particular stages of the circadian cycle that are maximally sensitive to a substance, drugs are often added for only a short period. When chemicals are pulsed in this way, the investigator looks for a change in the phase of the rhythm.

The effects of protein synthesis inhibitors on several organisms and rhythms have been explored using these techniques. Two generalizations about the effectiveness of these inhibitors are beginning to emerge: inhibitors of protein synthesis on 70S ribosomes, such as streptomycin and chloramphenicol, are usually ineffective (Hastings, 1960; Karakashian and Hastings, 1962, 1963; Sweeney *et al.*, 1967; Enright, 1971; Mergenhagen and Schweiger, 1975a), but inhibitors of protein syn-

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Abbreviations: + mating type, mt+; − mating type, mt−; high salt-concentration medium, HSM.

[3] Some of these experiments were initially done by Anthony Carter as part of a Senior Honors thesis (unpublished) at Princeton University.

thesis on 80S ribosomes, such as cycloheximide, puromycin, and anisomycin, do affect the period or phase of some rhythms (Karakashian and Hastings, 1963; Brinkmann, 1971, 1973; Feldman, 1967; Mergenhagen and Schweiger, 1975b; Karakashian and Schweiger, 1976a, 1976b; Rothman and Strumwasser, 1976, 1977; Jacklet, 1977; Straley and Bruce, 1979; Walz and Sweeney, 1979; Dunlap *et al.*, 1980). Therefore, one might tentatively infer either that 80S protein synthesis is involved in the mechanism of the clock or that protein components of the clock must be periodically resynthesized.

The results of other experiments are consistent with the suggestion that membrane structure or function is important to circadian time-keeping. If this is true, then protein synthesis may affect the clock indirectly by influencing the supply of a protein involved in active transport (Sargent *et al.*, 1976). The evidence that membranes are actually involved in the generation of circadian oscillations is mainly circumstantial. Substances such as ethanol (Keller, 1960; Bünning and Baltes, 1962; Bünning and Moser, 1973; Brinkmann, 1974, 1976; Sweeney, 1974; Taylor *et al.*, 1979), valinomycin, an ionophore of K^+ (Bünning and Moser, 1972; Sweeney, 1974, 1976), and ions such as K^+ (Eskin, 1972; Bünning and Moser, 1973) and Li^+ (Engelmann, 1972, 1973; Engelmann *et al.*, 1974; Eskin, 1977) modify some rhythms and are also known to influence membranes and/or transport.

Genetics also has been used to probe the mechanism of the clock. Genetic analysis has isolated mutants with altered clock properties in *Drosophila* (Konopka and Benzer, 1971; Pittendrigh, 1974), *Neurospora* (Feldman and Wasser, 1971; Feldman *et al.*, 1973; and Feldman and Hoyle, 1974) and *Chlamydomonas* (Bruce, 1972, 1974). In addition, mutations altering some aspect of physiology have been studied to determine whether the mutations also affected the circadian clock (Feldman, *et al.*, 1979).

Combining the chemical manipulation and genetic approaches has been informative. Clock properties can be compared in wild type organisms sensitive to a particular chemical and in organisms carrying a mutation for resistance to that chemical. Nakashima *et al.* (1981) have shown that in two *Neurospora* mutants whose growth is resistant to cycloheximide, the conidiation rhythms also are unaffected by the drug. When Dieckman and Brody (1980) crossed a mutation of resistance to oligomycin into the band strain of *Neurospora*, the period length decreased from the 21.5 h characteristic for the band strain to 18–19 h.

The most useful picture of the pattern of effects of chemicals emerges when a whole spectrum of inhibitors is tested on the circadian system of a single organism. *Chlamydomonas* is a unicell that has a well defined circadian rhythm in phototaxis (Bruce, 1970). This study tested effects of a variety of chemicals on this rhythm to identify cellular processes that may be important to circadian timing in this organism.

Materials and Methods

Culture techniques

Chlamydomonas reinhardii strains, mt^+ and mt^- (Bruce, 1970), as well as the clock mutants per 1 mt−, per 2 mt−, and per 4 mt^+ (Bruce 1972, 1974), and a cycloheximide resistant mutant (described below) were used in these experiments. Experimental cultures were grown on a shaker table under continuous illumination

from cool white fluorescent lamps (1500–3000 lux) at 22°C. The cultures were grown to a density of 1–2 × 10^6 cells/ml.

Phototactic assay

Phototactic rhythm assays were made as described previously (Bruce, 1970). Samples of the culture (1.5 ml) were placed in individual wells of plastic tissue-culture trays and the drugs were added to the final concentrations indicated at the beginning of each experimental test.

Cell counts

Samples of the culture were diluted 1:100 with 0.9% saline solution, and then cell counts were made with a Coulter counter. Unless otherwise specified, cultures were counted at 1 or 2 day intervals until high cell density limited growth.

Genetic analysis

The cycloheximide resistant mutant *cyr*-1 was isolated from mt− after exposure to the mutagen nitrosoguanadine (Bruce, 1972) and plating on 0.3 high salt-concentration medium (HSM) plates supplemented with 10 μg/ml of cycloheximide. Diploids of *cyr*-1 were constructed using arginine-requiring strains (Bruce and Bruce, 1978). They were confirmed to be diploids by crossing them with wt and recovering arginine-requiring progeny. Sensitivity or resistance to cycloheximide was determined by spot plating on plates supplemented with 10 μg/ml cycloheximide.

Chemicals

The following chemicals were tested on the phototactic rhythm:

Drug	Range
actinomycin (Merck, Sharpe, and Dohm)	0.25, 0.5, 1.0, 2.0, 4.0 μg/ml
rifampicin (CIBA)	0.25, 0.5, 1.0, 2.0, 4.0 μg/ml
chloramphenicol (Sigma)	2.75, 5.5, 11.25, 22.5, 45 μg/ml
streptomycin sulfate (Lily)	45.5, 60, 70 μg/ml
cycloheximide (Actidione) (Upjohn Co, Kalamazoo	0.05, 0.08, 0.1, 0.15, 0.2, 0.25, 0.5, 1, 4 μg/ml
puromycin dihydrochloride (Sigma)	1.3, 26, 130 μg/ml
valinomycin (Sigma)	0.09, 0.45, 0.83 μg/ml
LiCl (Fischer)	0.05, 0.09, 0.36, 0.45, 0.64 m*M*
procaine (Sigma)	0.33, 1.0, 3.3, 10 mg/ml
tetracaine (Sigma)	0.033, 0.1, 0.33, 1.0 mg/ml
dibucaine	0.0033, 0.01, 0.033, 0.1 mg/ml
sodium lauryl sulfate	0.0033, 0.01, 0.033, 0.1 mg/ml
ethanol	0.005, 0.18, 0.27%
methanol	0.1, 1.0, 2.0%
isopropanol	0.1, 1.0, 2.0%
chloral hydate	0.167, 0.5, 0.99, 1.67, 5.0 mg/ml

Results

Inhibitors of transcription

Actinomycin D (added in ethanol) in concentrations up to 2 μg/ml allowed phototaxis to be expressed but had no effect on the period length of the rhythm. At the highest concentration, phototaxis was inhibited. Growth was inhibited in a dose-related manner at all drug concentrations. The period length of ethanol controls was the same as that of controls in untreated media.

Rifampicin did not alter the period length of the phototactic rhythm and had no reliable effect on the growth rate.

Inhibitors of translation

Chloramphenicol did not affect either the phototactic rhythm or growth rate.

Streptomycin had no effect on the period length of the rhythm but eliminated rhythmicity in some samples after approximately 4 cycles. Higher concentrations killed the cells.

Puromycin dihydrochloride at low concentrations (1.3 and 26 μg/ml) had no effect on the phototactic rhythm, but a concentration of 130 μg/ml increased the period length 8.3%, from 24 to 26 h (Fig. 1). The growth rate was inhibited at all concentrations, but at lower concentrations the inhibition was not immediate.

Cycloheximide's period lengthening effect on the per 2 mt− strain is shown in Figure 2. The effect on the rhythm was immediate and dose-related. The period lengthening effects on per 2 mt−, per 4 mt^+ and wt mt− are summarized in Figure 3. Although cycloheximide consistently lengthened the period in all the experiments, some differences between strains were noted in different experiments, and the drug's effect did not always persist indefinitely. For example, 0.15 and 0.25 μg/ml of cycloheximide lengthened the period of the per 1 mt− mutant for the initial 2–3 cycles after exposure, but the period eventually returned to the same length as controls (Fig. 4).

Cell counts taken over several days, but at 3 h intervals during the first day, indicate that in all samples treated with 0.1, 0.2, 1, and 4 μg/ml cycloheximide, growth was inhibited for the first 12 h of exposure. After the first 12 h, all the

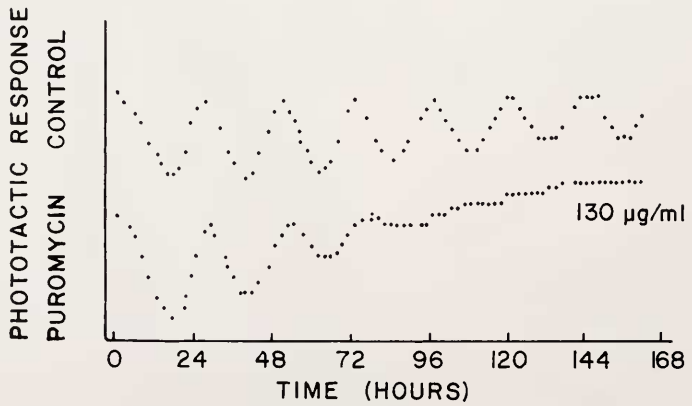

FIGURE 1. The rhythmic phototactic response of mt− strain of *Chlamydomonas reinhardii* and its modification by puromycin.

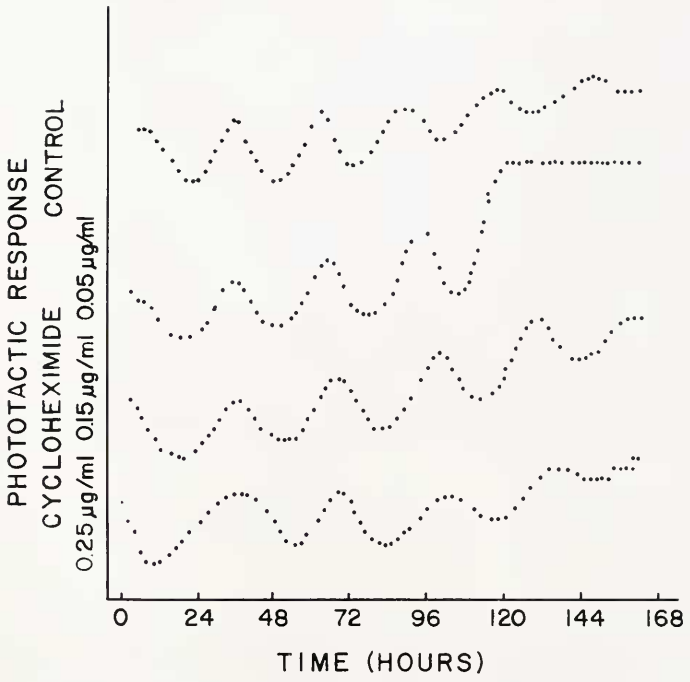

FIGURE 2. The rhythmic phototactic response of the mt− strain of *Chlamydomonas reinhardii* and its modification by cycloheximide.

period mutants treated with 0.1, or 0.2 µg/ml of cycloheximide had increased growth rates. At higher drug concentrations, the inhibition of growth lasted slightly longer. In cultures treated with 4 µg/ml, the cell growth that did occur ceased after the first 24 h. Cell numbers fell below the number counted on day 1 and cells were bleached.

Although cycloheximide lengthens the period of many mutants, this is not the case with the cycloheximide resistant mutant *cyr*-1, whose rhythm is unaffected by concentrations of cycloheximide which completely inhibit the response of the wild type. We have constructed diploids of *cyr*-1 and the wild type. Because of the way in which diploids are obtained in *Chlamydomonas*, they also contain arginine markers, in this case various combinations of arg 1, arg 2, and arg 7. All twelve diploids tested were sensitive to cycloheximide. The drug's effect on the period length of the diploid's rhythm was similar to that of the wild types. The average period lengthening effect of 0.5 µg/ml was 2.3 h (10 diploids) and of 1.5 µg/ml, 3.1 h (9 diploids).

Chloral hydrate did not affect the rhythms of mt− or the per 2 mutant at drug concentrations up to 0.99 mg/ml. The mt− strain was tested at 17° and 23°C.

"Membrane-active" chemicals

The alcohols methanol, ethanol, and isopropanol did not influence the phototactic rhythm at the concentrations tested.

Valinomycin and valinomycin (0.45 µg/ml) with additional K^+ ions (4.55 m*M* KCl) also did not alter the period length of the rhythm.

Li^+ ions in the form of LiCl did not alter the rhythm.

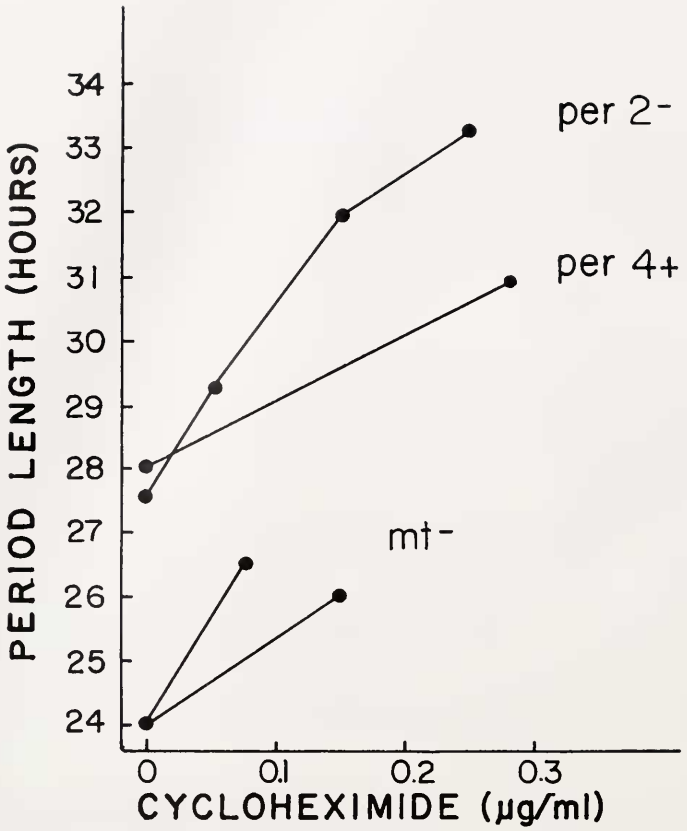

FIGURE 3. Period lengthening effects of cycloheximide on the wild type mt− and the mutants per 2 mt− and per 4 mt^+.

The local anesthetics, procaine, tetracaine and dibucaine had no effect on the rhythm when tested at concentrations 1/3, 1/10 and 1/30 times the minimum values that inhibited phototaxis.

DISCUSSION

Inhibitors of transcription and translation

Our survey of the effects of inhibitors of transcription and translation on the *Chlamydomonas* rhythm support the generalization from other clock systems that the aspect of gene expression important to the timing of circadian rhythms is the translation of proteins on the 80S ribosomes. This statement is based on the increase in the period length of the rhythm that results from sustained administration of cycloheximide and puromycin, chemicals that inhibit translation on the 80S ribosomes. Antibiotics that inhibit transcription, such as AcD and rifampicin, and those that inhibit translation on the 70S ribosomes, such as streptomycin and chloramphenicol, did not appear to slow the biological timing process. These data are consistent with the generalizations mentioned earlier that translation of proteins on the 80S but not the 70S ribosomes is essential to the timing of circadian rhythms.

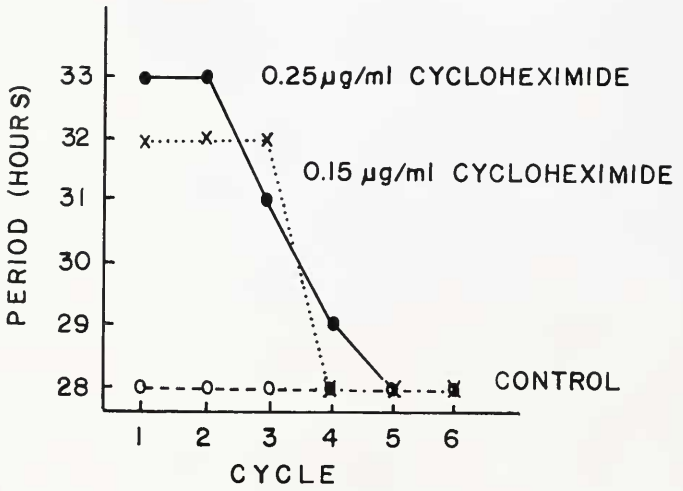

FIGURE 4. The effect of cycloheximide on the period length of the phototactic rhythm of the per 1 mt−.

To test whether particular chemicals did enter cells we measured the effect of the inhibitors on growth. The reason that AcD did not affect phototactic rhythm probably was not its inability to enter the cells, since concentrations that had no effect on the timing of the rhythm did inhibit cell growth. AcD is destroyed by light, which is essential to assay the phototactic response. However, the growth rate was inhibited by AcD in cultures grown in continuous light, so destruction of the inhibitor by light cannot explain the lack of effect on the circadian timing. On the other hand, rifampicin did not inhibit cell growth in cultures grown in continuous light or continuous darkness, so the question of whether it entered the cells remains.

Chloramphenicol also did not influence growth rate or the phototactic rhythm in these experiments. Jacobson *et al.* (1964) demonstrated that $3 \times 10^{-4}M$ chloramphenicol had an antimitotic effect on *Chlamydomonas reinhardii*, indicating the chemical can enter the cells. However, the drug concentrations used in the present experiment are below those used by Jacobson *et al.*, and therefore, may have been too low to diffuse to its sites of action, the mitochondria and chloroplasts. The second inhibitor of translation on the 70S ribosomes tried in this experiment, streptomycin, also did not alter the period length of the phototactic rhythm. We did not determine its effect on growth rate. However, the appearance of arrhythmicity in some of the samples treated with 45.5 µg/ml streptomycin, and the fact that higher drug concentrations killed the cells, suggest that this inhibitor was able to enter the cells, and that its failure to affect the timing of the clock is evidence that translation of the 70S ribosomes is not essential to the timing mechanism.

Both puromycin and cycloheximide, the inhibitors of translation on the 80S ribosomes tested in these experiments, slowed biological timing. Cycloheximide was effective at low concentrations, whereas the relatively high concentration of puromycin required to affect growth, as well as the rhythms, probably reflects the cells' relative impermeability to this drug.

Cycloheximide was previously shown to lengthen the period of the *Chlamydomonas* sticking rhythm (Straley and Bruce, 1979). In some of their experiments,

as well as in a few of the present experiments on phototactic rhythm, the clock was only affected by cycloheximide for 2–3 cycles before the rhythm began to return to the period length of the controls (Fig. 4). We do not know why this occurs. However, since the drug's effect on the growth rate of the cells was similar, it may reflect some change in the cells' ability to deal with cycloheximide, perhaps by an inducible enzyme that degrades cycloheximide or by changes in permeability.

Since the diploids were created from the combination of a strain resistant to cycloheximide and a wild type strain, the fact that all diploids were as sensitive to cycloheximide as the wild type indicates that the mutation for resistance (*cyr*-1) is recessive. The fact that cycloheximide does not alter the period of the resistant mutant *cyr*-1, but lengthens the period of the recessive diploid, suggests that this drug affects the rhythm because of its inhibition of protein synthesis, rather than via some secondary effect of cycloheximide. Our observations on *Chlamydomonas* are in agreement with those of Nakashima *et al.* (1981) with *Neurospora*. These workers found that the conidiation rhythms of two cycloheximide resistant mutants are unaffected by the drug. The site of cycloheximide resistance in the *Neurospora* mutants is known to be protein synthesis on 80S ribosomes.

Drugs commonly have more than one effect on living cells. Therefore, one can never be sure that a chemical that affects the clock is exerting its influence via its primary mode of action. One way to increase the certainty that a drug is acting through its assumed mode of action is to test other chemicals that affect the same process. In this study, more than one inhibitor of transcription, of translation on 70S ribosomes and of translation on 80S ribosomes, were tested for their effectiveness on the phototactic rhythm. The effects of inhibitors with similar primary modes of action were always the same.

Although translation on the 80S ribosomes seems essential to the functioning of the circadian clock controlling phototactic rhythm in *Chlamydomonas*, we do not know whether this translation is directly involved as a part of the timing process, or indirectly involved by influencing some other cellular component.

The membrane-active chemicals

Many workers have suggested that membrane structure and/or function may be the basis for circadian timekeeping (see Sweeney, 1976 for a review of the evidence). We have tried a variety of chemicals believed to have affected the structure and or function of membranes under the conditions of our experiments. None altered the period of the phototactic rhythm.

One approach in the attempt at altering membrane structure or function was the continuous administration of local anesthetics (procaine, tetracaine, and dibucaine) known to act at the membrane level, and of a detergent, sodium lauryl sulfate. None of these treatments altered the period length of the rhythm.

Ethanol alters membrane fluidity (Chin and Goldstein, 1976) and active transport (Kalant, 1971). Both these aspects of the membrane are important in the timing mechanism of the clock, according to the membrane hypothesis of Njus *et al.* (1974), which proposed that the clock's timing mechanism is a system of feedback between the concentration of an ion on one side of the membrane and the active transport of that ion across the membrane. Although other workers have found that ethanol may alter biological timing, its effect is not consistent on all systems. It increases the period length of the *Phaseolus* sleep movement rhythm (Keller, 1960) and the *Euglena* motility rhythm (Brinkmann, 1974, 1976) but it

shortens the period of the petal movement rhythm of *Kalanchoe* (Kastenmeier, *et al.*, 1977) and the glow rhythm of *Gonyaulax* (Taylor *et al.*, 1979). Our failure to find any effect on the period length of *Chlamydomonas* is one more inconsistent piece in the puzzle.

Brinkmann (1976) has noted that the effect of alcohols on biological timing in *Euglena* and in *Gonyaulax* (Sweeney, 1976) decreases with increasing carbon chain length. On the other hand, the effect of an alcohol on a membrane increases with carbon chain length. He suggested that the effect of alcohols on the clock may be through metabolism. In our experiments, methanol, ethanol, and isopropanol did not change the period of length.

Because ion transport has been suggested as an important component of the clock mechanism (Njus *et al.*, 1974) many workers have tried to alter biological timing with valinomycin, an ionophore of K^+ ions, or with the ions K^+ and Li^+. Phase changes from pulses of valinomycin (Sweeney, 1974, 1976) and K^+ (Eskin, 1972; Bünning and Moser, 1973) and period lengthening from Li^+ (Engelmann, 1972, 1973) have been reported. In contrast, however, the *Chlamydomonas* clock was unaltered by these chemicals.

Although planned pulsing experiments may elucidate points in the cycle that are affected by "membrane-active" chemicals, our results do not support the view that the membrane is important for functioning of the *Chlamydomonas* clock.

Acknowledgments

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